Mesenchymal Stem Cells Enhance Allogeneic Islet Engraftment in Nonhuman Primates

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Running title: MSC and islet engraftment in nonhuman primates

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Objective: To test the graft promoting effects of mesenchymal stem cells (MSC) in a cynomolgus monkey model of islet/bone marrow transplantation.

Research design and methods: Cynomolgus MSC were obtained from iliac crest aspirate and characterized through passage 11 for phenotype, gene expression, differentiation potential, and karyotype. Allogeneic donor MSC were cotransplanted intraportally with islets on post operative day (POD) 0 and intravenously with donor marrow on POD 5 and 11. Recipients were followed for stabilization of blood glucose levels, reduction of exogenous insulin requirement (EIR), c-peptide levels, changes in peripheral blood T regulatory cells and chimerism. Destabilization of glycemia and increases in EIR were employed as signs of rejection; additional intravenous MSC were administered to test effect on reversal of rejection.

Results: MSC phenotype and a normal karyotype were observed through passage 11. IL-6, IL-10, VEGF, TGF-β, HGF and galectin-1 gene expression levels varied between donors. MSC treatment significantly enhanced islet engraftment and function at 1 month posttransplant (n=8), as compared to animals that received islets without MSC (n=3). Additional infusions of donor or 3rd party MSC resulted in reversal of rejection episodes and prolongation of islet function in 2 animals. Stable islet allograft function was associated with increased numbers of T regulatory cells in peripheral blood.

Conclusions: MSC may provide an important approach for enhancement of islet engraftment, thereby decreasing the numbers of islets needed to achieve insulin independence. Furthermore, MSC may serve as a new, safe and effective anti-rejection therapy.

Multipotent mesenchymal stem cells (MSC) (1; 2) can deliver immunomodulatory signals (3-7) that inhibit allogeneic T cell responses through downregulation of the pro-inflammatory cytokines TNF-α and IFN-γ and production of the regulatory cytokines/molecules IL-10, hepatocyte growth factor (HGF), TGF-β, vascular endothelial growth factor (VEGF), indoleamine 2, 3, dioxygenase (IDO), galectin-1, prostaglandin E2, nitric oxide and matrix metalloproteinase (MMP) -2 and -9 (3; 8-12). Inflammatory signals, such as IFN-γ, can activate and up-regulate MSC suppressive activities (9; 13). These cells are able to migrate to sites of injury after intravenous injection (14; 15). Their use in clinical trials and experimental models is based on their immunomodulatory and regenerative properties (1; 7; 16). Clinically, MSC have been observed to enhance donor bone marrow cell (DBMC) engraftment and chimerism (17; 18). Therefore, cotransplantation of MSC that secrete immunomodulatory cytokines and growth factors might enhance islet survival and function. In experimental mouse models, intravenously infused MSC are capable of migrating to pancreatic islets (19; 20). Systemic infusion of MSC in murine models of diabetes was accompanied by delayed onset of diabetes, improved glycemic levels, reduced pancreatic insulitis and pancreatic tissue regeneration (19; 21-25), as well as prevention of autoimmune destruction of beta cells via induction of T regulatory cells (26). Cotransplantation of syngeneic MSC with a marginal mass of allogeneic islets under the kidney capsule of streptozotocin (STZ)-induced diabetic mice resulted in prolonged
normoglycemia (11). Cotransplantation of syngeneic MSC with a marginal mass of allogeneic islets has been performed in the omentum (27) and kidney capsule (28) of STZ-induced diabetic rats, with enhanced islet graft survival as compared to animals receiving islets alone. In this study, cynomolgus monkey MSC were characterized and donor MSC examined for ability to promote intraportal islet engraftment, as well as chimerism in recipients of islet/DBMC transplants. In addition, we tested the use of donor or 3rd party MSC to reverse episodes of islet allograft rejection.

RESEARCH DESIGN AND METHODS

Animals. Donor and recipient cynomolgus monkeys (>4 and >2 years of age, respectively) were obtained from Charles River BRF, Inc. (Houston, TX) or Alpha Genesis, Inc. (Yemassee, SC) and were negative for TB, Herpes B, SRV, SIV and STLV-1. Each donor-recipient pair was tissue typed retrospectively and demonstrated to be fully or partially mismatched for MHC Class II alleles identified using microsatellite analysis as previously described (29-31). All study transplant protocols were approved by The Institutional Animal Care and Use Committee of The University of Miami.

Diabetes induction and management, islet preparation and transplantation. Diabetes was induced with streptozotocin (STZ, 1,250 mg/m² i.v.) (32) and defined as fasting c-peptide (CP) <0.2 and stimulated CP <0.3 ng/ml in response to a glucagon challenge undertaken four weeks post STZ (32). Blood glucose levels were monitored 2-3 times daily via heel stick. Subcutaneous insulin was administered as needed, based on an individualized sliding scale, to maintain the following plasma glucose levels: 250-350 for the first 2 weeks post-STZ; 175-250 for the 3 and 4th weeks, and 75-150 mg/dl prior to and after transplantation. Using established methods, the donor pancreas was recovered, islets isolated and cultured for 39-42 hours, and islets were collected, washed and counted for transplantation into the liver as previously described (32). For group 1 (Table 1), islets were infused first, followed immediately by infusion of DBMC. For group 2 (Table 2), MSC were added to the islet preparation 15 to 20 min before intraportal infusion.

Isolation of donor hematopoietic stem cells. Donor vertebral bodies were harvested and processed to obtain DBMC as previously described (33). DBMC were depleted of CD11b positive cells using EasySep magnetic cell separation (Stem Cell Technologies, Vancouver, BC), cryopreserved in cryoMACS freezing bags (Miltenyi, Auburn, CA) at a concentration of 500 x 10⁶ cells/bag and stored until infusion.

Isolation, culture and expansion of MSC. Bone marrow aspirates were harvested from the iliac crest of donor or 3rd party monkeys and processed with Ficoll Paque Plus to obtain mononuclear cells. Cells (P0) were plated at a density of 5 x 10⁷ cells per 185 cm² Nunclon Delta Solo flask (VWR, West Chester, PA) in culture media consisting of Minimum Essential Medium Alpha Media (Invitrogen) supplemented with 20% FBS (Hyclone, Logan, UT), 1% Penicillin-Streptomycin (Invitrogen) and 1% L-glutamine (Mediatech). Cells were kept in culture at 37ºC, 5% CO₂ with two media changes weekly. Once cells reached confluency, the adherent cells were removed using 0.25% Trypsin-EDTA (Invitrogen) (37ºC for 5 minutes). P1 and P2 cells were plated at a concentration of 1 x 10⁶ cells per flask; subsequent passages were plated at 10⁷ cells/ml; cells were cryopreserved in 90% FBS and 10% dimethylsulfoxide (Sigma).

Characterization of MSC. Differentiation: Following manufacturer’s instructions (Human Mesenchymal Stem Cell Functional Identification Kit, R & D Systems, Minneapolis, MN), MSC were characterized for osteogenic and adipogenic differentiation.
Immunomodulatory capacity: P2 MSC (5x10⁴ cells) were allowed to adhere for 24 hours to a U-bottomed 96 well plate (Corning, New York), followed by addition of responding PBMC (1x10⁵/well) and phytohemagglutinin (PHA) at a final concentration of 10 µg/well, in 0.2 ml of culture media. Cultures were incubated at 37°C, 5% CO₂ for 5 days and T cell proliferation was determined by addition of [³H]thymidine (GE Healthcare) at 1 µCi/well for the last 18 hrs of culture, harvesting onto fiberglass filters and counting. Karyotyping: Adherent MSC were treated with Colcemid™ (0.1µg/ml) for two hours prior to cell harvesting. Following mitotic arrest, cells were processed in accordance with standard cytogenetics laboratory procedures at the University of Pittsburgh Cancer Institute Cytogenetics Facility (Pittsburgh, PA). Gene expression: Total RNA was isolated from MSC using an RNeasy kit (Qiagen, Valencia, CA). First strand cDNA was synthesized using ‘SuperScript III First-Strand Synthesis SuperMix for qRT-PCR’ kit according to manufacturer’s instructions (Invitrogen). Gene expression levels for IL-6, IL-10, HGF, VEGF, TGF-β and galectin-1 were determined by using Taqman® assays (Applied Biosystems, Foster City, CA) in the LightCycler PCR system (Model 1.2, Roche). Taqman® assay IDs for the genes are IL6: Hs00985639_m1; IL-10: Hs00174086_m1; HGF: Hs00300159_m1; VEGF: Rh02621759_m1; TGF-β: Hs00171257_m1; galectin-1: Hs00169327_m1; and 18S: Hs99999901_s1. Amplification of each sample was performed in a 20 µl reaction mixture containing 1X LightCycler FastStart DNA Master HybridizationProbe (Roche Diagnostics, Mannheim, Germany), 1X Taqman® assay, 4 mM MgCl₂, and 2 µl cDNA sample. PCR amplification consisted of 95°C for 10 min, followed by 40 cycles at 95°C for 10 s and at 60°C for 1 min. DNA fragments from each target gene (from copy number 10⁹ to 10²) were used to construct the standard curve in each PCR amplification. Results are expressed as the ratio of the copy number of the target gene to the copy number of 18S.

Flow cytometry analyses. MSC: 10⁵ cells were labeled with conjugated monoclonal antibodies specific for CD14, CD29, CD56, CD90, HLA Class II (Beckman Coulter , Fullerton, CA), CD11c, CD34, CD44, CD45, CD73, CD166, HLA Class I (BD PharMingen, SanDiego, CA), CD31 (Ebioscience, San Diego, CA) and CD105 (Fitzgerald Industries International, Concord, MA). Whole blood: 100 µl of EDTA blood was labeled with a combination of monoclonal antibodies (mAbs) specific for CD3, CD4, CD45 (BD Pharmingen), CD25 (eBioscience), CD8, CD11b, CD16, CD20, CD56, CD69, CD127 and HLA DR (Beckman Coulter); 7AAD was included for viability assessment. Erythrocytes were lysed using an ImmunoPrep Reagent System and a Q-Prep Workstation (Beckman Coulter). Foxp3: Following manufacturer’s instructions, cynomolgus PBMC were stained intracellularly for Foxp3, clone PCH101 (eBioscience). All samples were analyzed on a Coulter Cytomics FC500.

Experimental design, immunosuppressive regimen, and drug levels. Fig. 4A is a schematic of the design used to test the effect of intraportal codelivery of islets with donor bone marrow cells (DBMC; Group 1; Table 1) or with donor MSC (Group 2, Table 2) on chimerism and islet allgraft survival. We depleted CD11b positive vertebral body bone marrow cells in order to debulk the marrow and remove fragile myeloid cells. Myeloid cell death results in DNA release, clumping and decreased overall cell viability (34). A total of 3 animals in Group 1 received induction therapy consisting of four 10 mg/kg doses of thymoglobulin and 4 doses (50 mg/m² total) of fludarabine on POD -6, -4, -3 and -2. IM rapamycin was initiated on POD -2 to achieve and maintain trough levels of 15-
20 ng/ml. In this group, we examined the effect of intraportal codelivery of islets and DBMC on POD 0, followed by IV infusions of DBMC on POD 4 or 5 and 11. The timing of DBMC infusion was based on previous clinical studies, in which delayed marrow infusion was found to be optimal in the setting of solid organ or islet transplantation (35). Group 2 had 8 animals that received the same induction therapy with thymoglobulin and fludarabine. In 5/8 animals, IM rapamycin was initiated on POD -1 to achieve and maintain trough levels of 15-20 ng/ml. Rapamycin was delayed (POD 14) for the remaining 3 animals (105-131, 26-20 and CW1H), which were also treated with 20 mg/kg human/mouse chimeric anti-CD154 specific monoclonal antibody, derived from the 5c8 clone (NCRR Nonhuman Primate Reagent Resource), on POD -1, 0, 3, 10, 18, 28 and monthly thereafter. The noticeable lack of chimerism observed after the first 2 animals prompted the addition of parathyroid hormone (PTH) to augment chimerism in the subsequent 6 animals (Forteo, 5 ug/kg from POD -7 or -6 until POD 49). All animals in group 2 were used to examine the effect of intraportal codelivery of islets and MSC on POD 0, followed by IV infusions of CD 11b depleted DBMC + MSC on POD 5 and 11.

**Chimerism.** The levels of donor DNA in a recipient peripheral blood sample were determined twice/month using LightCycler PCR as previously described (37). Chimerism results were reported as donor percentages, and each sample was analyzed in duplicate.

**Histopathology.** Tissues fixed in 10% neutral buffered formalin and embedded in paraffin were sectioned (5 μm) and stained with hematoxylin and eosin (H&E). Insulin expression in the islets was assessed by immunohistochemistry using a guinea pig anti-porcine insulin polyclonal antibody (Dako, Carpinteria, CA) and a biotinylated donkey anti-guinea pig immunoglobulin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), followed by streptavidin-horseradish peroxidase and revealed by aminoethylcarbazole (Invitrogen, Carlsbad, CA). For immunofluorescence microscopy, sections were stained with anti-insulin (AbCam, Cambridge, MA), anti-glucagon (Sigma, St. Louis, MO), and anti-CD31 (AbCam), anti-CD34 (BioGenex, San Ramon, CA) and anti-actin (AbCam) for blood vessels followed by Alexa Fluor conjugated secondary antibodies (Molecular Probes, Carlsbad, CA) as previously described (38).

**Statistics.** All data represent means ± SEM. A repeated measures ANOVA was used to evaluate within-group c-peptide values at different time points posttransplant, as well as gene expression levels at different MSC passages. The between-group comparison of c-peptide values at each time point posttransplant was evaluated using a t-test. In all appropriate cases, post hoc LSD test was used. All analyses were performed using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL) and p values < 0.05 were considered statistically significant.

**RESULTS**

**Isolation, expansion and characterization of cynomolgus monkey MSC.** Vertebral body marrow and iliac crest aspirate (ICA) were evaluated as the MSC source and ICA was optimal with regards to cell yield. Cells were characterized to verify absence of cell surface markers associated with leukocytes (CD11c, 14, 45, 56), endothelial and hematopoietic stem cells (CD31, 34), and HLA DR and presence of cell surface markers associated with MSC in published studies, including CD29, 44, 73, 90, 105 and 166, as well as class I. The percentage of non-MSC associated markers was highest in the source material, much lower in P1, and dropped to less than 1% thereafter, with a corresponding increase in the percentage of CD105, 29 and
73 cells to nearly 100% (Figure 1A and B). Markers reported on MSC from other species (CD90, 44, 166), as well as CD56, were variably positive. Differentiation of cells to fat and bone verified MSC identity (Figure 1C). Addition of MSC to cynomolgus mixed lymphocyte reactions resulted in variable suppression of proliferation (data not shown). In contrast, addition of either autologous (n=2) or allogeneic (n=13) MSC to PHA stimulated PBMC significantly inhibited proliferation by 85% (Figure 1D, p<0.009).

We assessed gene expression levels for IL-6, IL-10, VEGF, TGF-β, HGF and galectin-1 over several passages of MSC from the same donor, with peripheral blood as a control; 8 different donors were studied through passage 5 (Figure 2) and 4 through passage 11 (data not shown). Levels of TGF-β remained stable through passage 8 and were similar to PBL and P0 material; significant decreases were noted after P8 for those cells followed further out. HGF, galectin-1, IL-6 and VEGF expression levels were significantly higher in MSC as compared to PBL and marrow and remained relatively stable through P5, with VEGF dropping off at P4, HGF and galectin-1 at P7 and IL-6 at P9. In contrast, IL-10 expression levels were extremely low, relative to control, in all passages. Variability in expression levels was observed between different donors. MSC from 4 of these animals were also characterized for cytogenetic stability at passages 0, 2, 6 and 11. Results revealed normal karyotypes, without clonal numerical or structural aberrations. The cells maintained clonal chromosomal stability between passages 0 and 11 (Figure 3).

**Effect of MSC on islet engraftment/function and chimerism:**

Figure 4A shows a schematic of the design used to test the effect of intraportal islet/donor bone marrow (DBMC) (group 1) and intraportal islet/MSC (group 2) transplant on chimerism and allogeneic islet engraftment. Islet function was detected for all recipients, as evidenced by stabilization of BG levels, decreased exogenous insulin requirements and positive fasting c-peptide. Attainment and duration of insulin independence was associated with islet dose (Tables 1 and 2).

Three animals in group 1 received intraportal islet/DBMC cotransplants on POD 0 (Table 1, and IV DBMC on POD 4 or 5 and 11. None of the group 1 animals experienced long term graft survival, with all recipients manifesting decreased graft function on POD 21-25. Insulin independence was transiently observed for the 2 monkeys that received a dose near the 10,000 IEQ/kg required for reproducible insulin independence in this model. Donor derived cells were only detected in peripheral blood in relation to DBMC infusion (POD 1-11).

Group 2 animals (Table 2) were treated with MSC, delivered both intraportally with the islets on POD 0 and IV with the delayed DBMC infusions. All animals in group 2 received 5 million intraportal MSC on POD 0, but due to the broad range of IEQ transplanted, this resulted in islet cell:MSC ratios from 3.7 to 13.4 (based on an IEQ of 1500 cells per islet). In contrast to group 1, decreased graft function was not evident until ≥ POD 60 for group 2 (as compared to POD 21-25 for group 1), with 5/8 animals c-peptide positive at necropsy. Mean duration of function prior to destabilization was 81±20 days for group 2 vs 24±2 for group 1 (p<0.001). This is illustrated in Figure 4B, which shows fasting blood glucose, exogenous insulin requirements and fasting c-peptide levels for one animal from group 1 (105-111; 11,598 IEQ/kg) and one animal from group 2 (35-493; 10,978 IEQ/kg) that received a comparable number of islets. Extremely low levels of chimerism, not associated with DBMC infusion, were observed transiently in 3/8 group 2 monkeys at approximately one month post transplant.
There was no significant difference in the mean number of islets transplanted in recipients from group 1 (9,774 ± 1,039 IEQ/kg; n=3) vs recipients from group 2, (7,412 ± 1,359 IEQ/kg; n=8). However, we observed a striking early increase in islet function in recipients of intraportal islet/MSC cotransplants, and chose 3 time points to compare islet function between animals in group 1 (intraportal islets/DBMC) vs group 2 (intraportal islets/MSC): 3 days, 2 weeks and one month posttransplant. We first determined that within the 8 animals in group 2, there was no significant difference in the fasting c-peptide values between the ones treated with delayed rapamycin and anti-CD154 (n=3) vs those that began treatment with rapamycin before transplant (n=5), or between the animals that received PTH (n=6) vs those that did not (n=2), or between the animals that were MHC class II mismatched (n=4) or haploidentical (n=4), at any of the 3 time points (data not shown). All 8 animals in group 2 were therefore used for subsequent analysis. The results for the comparison of fasting c-peptide levels at 3 days, 2 weeks and 1 month posttransplant between animals in group 1 and group 2 are shown in Figure 4C. Analysis of fasting c-peptide within each group as a function of time showed a trend towards an increase in function (p = 0.07) over the first posttransplant month only in the animals that received intraportal islet/MSC. Values for intraportal islet/DBMC recipients (group 1) did not change significantly in this same time frame. Comparison of c-peptide values between the 2 groups at 1 month posttransplant showed a significantly higher c-peptide value (more than double), for recipients of islet/MSC cotransplants (3.6 ± 0.5 ng/ml vs 1.4 ± 0.4 ng/ml, p = 0.043; Fig. 4C).

**Treatment of rejection with IV MSC and immunological changes in MSC treated recipients:** We used donor and/or 3rd party MSC to treat the rejection process after intraportal islet/MSC transplant in 5 of the animals in group 2 (93-108, 105-71, CW1H, 26-20, 105-131). Two animals received additional donor MSC (up to 2x10^6 MSC/kg) > 10 days after graft dysfunction (93-108 received one dose and CW1H received 2 doses administered 11 days apart) and one animal received a single dose of MHC class II mismatched 3rd party MSC (105-71) at the first sign of rejection without success. Our first evidence of efficacy in amelioration of rejection was obtained for an animal that experienced a decrease in function on POD 64 (animal 26-20, Table 2; Figure 5). This animal was treated with islet donor MSC (2 x 10^6/kg, MHC Class II mismatched to the recipient) on POD 64 and 68, but the EIR continued to rise. Infusion of 3rd party MSC (2 x 10^6 cells/kg, MHC Class II haploidentical to the recipient) on POD 71, 77, 86 and 91 resulted in EIR reduction and increased c-peptide, although the effect was short lived. Additional 3rd party MSC were given on POD 155 and 160, and at the time of necropsy, both fasting blood glucose and EIR were decreasing.

Therapy of an additional recipient with islet donor MSC, haploidentical to the recipient, with greater duration of follow-up post-MSC infusion, revealed clear reversal of rejection (105-131, Table 2, Figure 6). Figures 6A-D illustrate key points related to graft function, MSC infusion, T regs and T effector cells. This animal received a very marginal mass of 3,000 IEQ/kg. Posttransplant, a gradual reduction in insulin/kg was eventually offset by increased insulin requirements and destabilization of blood glucose on POD 94, accompanied by decreased fasting c-peptide (Figure 6A, lower panel). Additional islet donor MSC were given IV at 2x10^6/kg on POD 105, 110, 196 and 207. Of note is the gradual decline in insulin requirement after MSC infusion on POD 105 and 110 and the ultimate recovery of fasting c-peptide levels; additional infusions were given on POD 196.
and 207 with the rationale that repeated doses would have an additive effect. Exogenous insulin requirements, fasting blood glucose, as well as the day of additional MSC infusion (arrows) are shown in Figure 6A. Graft destabilization on POD 94 was associated with increased CD3/8 effector cells and decreased FoxP3 regulatory T cells (Tregs) (Fig. 6B). In addition, MSC infusion after resolution of graft dysfunction resulted in an increase in Tregs (Fig. 6B and C). Subsequent to graft recovery and additional IV MSC infusion, Tregs increased in percentage and absolute numbers to levels that were higher than those observed prior to rejection. Results from histological examination of tissues after necropsy are shown in Fig. 6D. Examination of liver sections using immunohistochemistry, as well as immunofluorescence staining, revealed insulin positive, highly vascularized scattered islets. Consistent with our previous observations in STZ-induced diabetic monkeys, islets in the pancreas were negative for insulin staining.

We measured the frequency of Tregs in 5 animals in group 2. Representative data for 2 animals, shown in Figs. 6 B and C (105-131) and Fig. S1 in the online appendix (35-493) (available at http://diabetes.diabetesjournals.org), illustrate that stable islet allograft function was associated with an increased number of Tregs in the periphery, while a decrease in Tregs occurred after graft dysfunction.

DISCUSSION
The International Society for Cellular Therapy criteria for human MSC include fibroblast-like morphology, adherence to plastic, phenotypic characteristics, \textit{in vitro} potential for tri-lineage differentiation and inhibition of proliferation of allo- or mitogen-activated lymphocytes (39). Rhesus (40) and cynomolgus macaque (41) and baboon (42) MSC appear to be phenotypically and functionally similar to their human counterparts. Our study depicts the first thorough characterization of cynomolgus MSC phenotypic markers, gene expression levels, and karyotype through several passages. Gene expression levels for IL-6, IL-10, VEGF, TGF-β, HGF and galectin-1 vary over several passages of MSC from the same donor (as well as between donors), indicating that the actual functional capacity of the cells might vary between donors and between passages. These findings could impact clinical outcomes and suggest a need for additional functional criteria in the standardization of MSC products for cell therapies.

The cytogenetic stability of these cells, studied up to passage 11, appear to be in agreement with human studies by Bernardo and colleagues (43), in which cells from 10 human donors, followed to senescence or passage 25, showed no cytogenetic abnormalities or malignant transformation. While small in numbers, our study demonstrates the feasibility of using cynomolgus macaque MSC up to passage 11 for therapeutic efficacy in future studies. MSC have been shown to enhance DBMC engraftment (17; 18). Studies in rodent models suggest that an intraportal route of antigen delivery may be tolerogenic (44; 45). Despite intraportal infusion of MSC or DBMC with islets, we did not observe enhancement of donor marrow engraftment, chimerism or tolerance. We did observe significant enhancement of islet engraftment and function at 1 month post transplant in recipients of intraportal MSC as compared to animals that received islets plus DBMC. To further define the significance of the engraftment data with MSC, we compared 1 month postransplant fasting c-peptide values from the animals that received intraportal islet/MSC (3.6 ± 0.5 ng/ml) vs 2 additional historical, published groups. The first group consisted of 3 recipients of intraportal islets
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alone, treated with thymoglobulin, fludarabine and rapamycin, plus samarium lexidronam and delayed IV DBMC on POD 5 and 11 (1.3 ± 0.2 ng/ml) (46). The second group was composed of 19 recipients of intraportal islets alone under the cover of steroid-free immune suppression (1.6 ± 0.2 ng/ml) (38). Taken together, and including animals from group1 in the present paper, it is striking that recipients of islets/MSC had engraftment values of more than double compared to 27 animals that did not get MSC, regardless of the conditioning regimen (p < 0.01). This suggests that compared to other regimens, MSC provide a distinctive advantage in promoting early islet engraftment. Marrow MSC have been shown to express chemokine receptors that mediate migration of these cells to pancreatic islets (20); after intracardiac infusion of MSC in diabetic mice, cells homed selectively to pancreatic islets and glomeruli (21) and IV infusion of MSC was accompanied by improved glycemic levels, reduced pancreatic insulitis and pancreatic tissue regeneration (19; 21-23; 25). While this data may suggest that the mechanism behind the engraftment effect of MSC observed in our NHP islet allograft model may involve migration of MSC to the pancreas and pancreatic islet regeneration, we did not observe any insulin positive islets in histological analysis of pancreas tissue after necropsy. We can hypothesize that MSC enhance islet engraftment by staying in proximity to the islets at the time of cotransplant, providing immunomodulatory, revascularization and regenerative signals. However, understanding the mechanism of action of MSC requires knowing where the MSC localize after intraportal or IV infusion. In an attempt to track the fate of the implanted MSC, we performed pilot experiments in which each of 2 monkeys was transplanted with intraportal islets/GFP-tagged MSC on POD 0 under the cover of anti-CD154. One month postransplant, the animals were euthanized for histological examination of the tissues. Using immunohistochemistry, as well as measuring GFP DNA, we could not find any traces of the GFP-tagged MSC in any of the tissues of one of the animals, but we could detect some GFP-tagged MSC in the liver and in the lungs of the other recipient (data not shown). Further experiments, including tracking tagged MSC after shorter periods posttransplant will be needed to elucidate the fate and potential mechanisms of action for intraportally implanted MSC in our NHP model of islet transplantation.

Intraportal islet engraftment and stable function were accompanied by an increase in the percentage of Tregs in the circulation. The immunomodulatory effect of MSC has been shown to involve generation of T regs in in vitro studies (47; 48), as well as in the prevention of autoimmune diabetes in NOD mice (26); however, our data is the first evidence of in vivo T reg generation in the context of MSC augmented allogeneic islet grafts in a preclinical model with close proximity to humans.

To the best of our knowledge, this is the first time that IV infusion of either islet donor or 3rd party marrow MSC were used to reverse episodes of islet graft rejection in the context of allogeneic islet transplantation. The most striking result was obtained after IV infusion of 2 doses of 2 x 10^6 islet donor MSC/kg spaced 4 days apart, given after the EIR had reached levels similar to those previous to transplantation of a marginal islet mass. The resolution of graft dysfunction was accompanied by an increase in the circulating Tregs. Timing appeared to be an important factor since MSC given several days after graft destabilization were ineffective. These findings suggest that the inflammatory process during rejection may provide activating cytokines or chemokines that enhance efficacy of MSC suppression. In another in vivo model of T cell mediated
tissue destruction, IFNγ was critical for suppression of graft versus host disease by MSC (49).
The use of a histocompatibility matched, autologous or a mismatched source of MSC when treating autoimmune disorders is still in debate. Fiorina et al. (24) reported delayed onset of diabetes as well as reversal of hyperglycemia in NOD mice treated with allogeneic but not autologous MSC, while Solari et al. (27) observed prolonged survival of allogeneic islets in STZ-induced diabetic rats with autologous but not with allogeneic MSC. The immunogenicity and functional integrity of MSC from subjects with autoimmune diseases is also not clear. Transplantation of autologous MSC in NOD mice was accompanied by development of soft tissue and visceral tumors, which were not observed with the transfer of allogeneic MSC (24). A concern with MSC from patients with autoimmune disease has been the potential for decreased immunomodulatory capacity, although recent studies reported that MSC from patients with multiple sclerosis and rheumatoid arthritis had normal cell surface and molecular phenotype and ability to support hematopoiesis (50; 51). It is not clear whether the capacity of these MSC is as robust as allogeneic MSC for immunomodulation or whether these cells require additional manipulation ex vivo to augment their immunomodulatory capacity.

Our results, obtained in a preclinical model, justify the clinical investigation of MSC as both a feasible approach for enhancement of islet engraftment and as a safe and effective anti-rejection therapy.

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REFERENCES
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Table 1. Intraportal cotransplant of allogeneic donor bone marrow cells and islets

<table>
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<tr>
<th>Group 1 ID #</th>
<th>MHC class II match</th>
<th>IEQ/kg</th>
<th>&quot;aDays off insulin&quot;</th>
<th>&quot;bDecrease in function&quot;</th>
<th>Days c-peptide positive/POD necropsy</th>
<th>&quot;cDose DBMC/kg x 10^6&quot;</th>
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<tr>
<td>93-343</td>
<td>MM</td>
<td>9,724</td>
<td>9</td>
<td>25</td>
<td>45/45</td>
<td>0.48</td>
<td>62.2</td>
<td>1, 5</td>
<td>~2</td>
</tr>
<tr>
<td>105-111</td>
<td>MM</td>
<td>11,598</td>
<td>12</td>
<td>21</td>
<td>46/61</td>
<td>0.43</td>
<td>71.5</td>
<td>4</td>
<td>0.37</td>
</tr>
</tbody>
</table>

Animals received induction treatment with 4 doses of 10 mg/kg thymoglobulin given in the week prior to transplant and 4 doses of 50 mg/m^2 total of fludarabine. IM rapamycin was initiated 1-5 days prior to intraportal islet/donor bone marrow cells (DBMC) transplant on POD 0, with a goal of achieving trough levels of 15-20 ng/ml. DBMC were debulked with a CD11b bead based method. Chimerism was measured using 6 class II based primer and probe sets for donor DNA, lower limit of detection ranged from 0.03-0.1%, depending on the primer probe set; ND = not determined; MM=recipient and donor are MHC class II mismatched; "a"not necessarily consecutive to islet transplant; "b"based on blood glucose levels, insulin requirement and/or c-peptide; "c"total DBMC dose; DBMC were given intraportally on POD 0 and IV on POD 5 and 11.
### Table 2. Intraportal cotransplant of allogeneic donor MSC + islets

<table>
<thead>
<tr>
<th>Group 2 ID #</th>
<th>^aMHC class II match</th>
<th>IEQ/kg</th>
<th>^bDays off insulin</th>
<th>^cDecrease in function</th>
<th>Days c-peptide positive/POD necropsy</th>
<th>^dDose DBMC/ kg x 10⁹</th>
<th>Dose CD34/ kg x 10⁶</th>
<th>POD 0 MSC/ kg x 10⁶</th>
<th>^eIV MSC/ kg x 10⁶</th>
<th>^fPOD chim</th>
<th>Peak Level (%) chim</th>
</tr>
</thead>
<tbody>
<tr>
<td>^a105-131</td>
<td>haplo</td>
<td>3,000</td>
<td>3</td>
<td>94</td>
<td>354/354</td>
<td>0.17</td>
<td>19.3</td>
<td>3.4</td>
<td>38</td>
<td>&lt;0.06</td>
<td></td>
</tr>
<tr>
<td>^a26-20</td>
<td>MM</td>
<td>3,928</td>
<td>0</td>
<td>64</td>
<td>202/202</td>
<td>0.20</td>
<td>23.9</td>
<td>1.6</td>
<td>5.3</td>
<td>UD</td>
<td>UD</td>
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<tr>
<td>105-117</td>
<td>haplo</td>
<td>4,581</td>
<td>0</td>
<td>60</td>
<td>60/89</td>
<td>0.31</td>
<td>63.0</td>
<td>1.5</td>
<td>6.2</td>
<td>UD</td>
<td>UD</td>
</tr>
<tr>
<td>93-108</td>
<td>MM</td>
<td>5,432</td>
<td>5</td>
<td>60</td>
<td>95/137</td>
<td>0.27</td>
<td>57.0</td>
<td>1.2</td>
<td>5.5</td>
<td>31</td>
<td>0.08</td>
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<td>^aCW1H</td>
<td>haplo</td>
<td>8,185</td>
<td>0</td>
<td>103</td>
<td>251/251</td>
<td>0.11</td>
<td>12.2</td>
<td>1.1</td>
<td>5.1</td>
<td>UD</td>
<td>UD</td>
</tr>
<tr>
<td>105-71</td>
<td>haplo; hmz</td>
<td>9,192</td>
<td>89</td>
<td>90</td>
<td>97/97</td>
<td>0.25</td>
<td>35.3</td>
<td>1.2</td>
<td>5</td>
<td>UD</td>
<td>UD</td>
</tr>
<tr>
<td>35-493</td>
<td>MM; hmz</td>
<td>10,978</td>
<td>106</td>
<td>106</td>
<td>181/181</td>
<td>0.30</td>
<td>60.4</td>
<td>1.4</td>
<td>5.8</td>
<td>UD</td>
<td>UD</td>
</tr>
<tr>
<td>105-99</td>
<td>MM</td>
<td>14,000</td>
<td>59</td>
<td>68</td>
<td>75/76</td>
<td>0.33</td>
<td>59.8</td>
<td>1.6</td>
<td>6.5</td>
<td>31</td>
<td>&lt;0.06</td>
</tr>
</tbody>
</table>

Animals received induction treatment with 4 doses of 10 mg/kg thymoglobulin given in the week prior to transplant and 4 doses of 50 mg/m² fludarabine. IM rapamycin was initiated 1-5 days prior to intraportal islet/donor MSC transplant on POD 0, with a goal of achieving trough levels of 15-20 ng/ml in all animals, except for 105-131, 26-20 and CW1H, in which rapamycin treatment started on POD 14. These 3 animals were also treated with anti-CD154. Chimerism was measured using 6 class II based primer and probe sets for donor DNA; ^aanti-CD154 rx; ^bMM=recipient and donor are MHC class II mismatched, haplo=MHC class II haploidentical, hmz=homozygous recipient; ^cnot necessarily consecutive to islet transplant; ^dbased on blood glucose levels, insulin requirement and/or c-peptide; ^eanimal expired CMV positive; ^fDonor bone marrow cells (DBMC) were debulked with a CD11b bead based method and given IV on POD 4 or 5 and 11; ^gMSC given IV on POD 5 and 11; ^chimerism, monitored twice per month, determined using 6 class II based primer probe sets for donor DNA, lower limit of detection ranged from 0.03-0.1%, depending on the primer probe set, UD=undetectable.
Figure Legends

Figure 1: A. Phenotypic characterization of MSC isolated from cynomolgus bone marrow. P0: aspirate; P1-P4: after 1st, 2nd, 3rd and 4th trypsinization, respectively. Values represent mean ± SD, number of different donors for each passage in parentheses. P0 (n=29); P1 (n=25); P2 (n=24); P3 (n=14) and P4 (n=9). B. Representative flow cytometric analysis of P2 cultured MSC. Compared to isotype control (dashed lines), cynomolgus bone marrow MSC stained positive for CD105, MHC class I, CD29 and CD73 and negative for CD45 and CD31. Histograms represent consistent findings in 24 different donors. C. Adipogenic and osteogenic differentiation of MSC (passage 2) isolated from cynomolgus bone marrow aspirates. D. Effect of MSC on phytohemaglutinin (PHA) induced stimulation of peripheral blood mononuclear cell proliferation. Allogeneic MSC significantly inhibited PBMC proliferation by 85% (p<0.009); results for autologous cells were similar but too few donors were tested to assess statistical significance.

Figure 2. Gene expression levels for IL-6, IL-10, VEGF, TGF-β, HGF and galectin-1 for sequential passages from 8 cynomolgus monkey donors. PBL: peripheral blood; P0: bone marrow aspirate; P1-P5: after 1st, 2nd, 3rd, 4th and 5th trypsinization, respectively. All results were expressed as the log ratio of the copy number of the target gene to the copy number of 18S (used as the endogenous control gene). Gene expression levels for PBL, P0 and P2-5 were compared to levels expressed in P1 MSC. n = 8, * p < 0.05 vs P1.

Figure 3: Trypsin-Giemsa banded karyotypes for mesenchymal stem cells from animal 105-74 from passages 0, 2, 6, and 11 showing a 42,XY normal male Pearson classification (52).

Figure 4: A. Schematic of the design used to test the effect of intraportal codelivery of islets with donor bone marrow cells (DBMC; Group 1) or with donor MSC (Group 2) on chimerism and islet graft survival in 2 groups of animals. Animals in Group 1 received induction therapy consisting of four doses of thymoglobulin (Thy) and 4 doses of fludarabine (Flu) on POD -6, -4, -3 and -2. IM rapamycin (Rapa) was initiated on POD -2. Islets were cotransplanted with DBMC intraportally on POD 0, followed by IV infusions of DBMC on POD 5 and 11. Animals in group 2 received the same induction therapy with Thy and Flu. a anti-CD154 (5C8) on POD -1, 0, 3, 10, 18, 28 and monthly thereafter and rapamycin was initiated on POD 14 (n=3); b parathyroid hormone (PTH) from POD -7 or -6 until POD 49 (n=6); c rapamycin was initiated on POD -1 (n=5). B. Exogenous insulin requirements (EIR), fasting blood glucose (FBG) (upper panel) and fasting c-peptide (lower panel) for representative animals from group 1 and group 2 that received similar number of islets. Both animals received induction therapy with thymoglobulin and fludarabine in the week prior to transplant and IM rapamycin starting on POD -1. The animal from group 1 (105-111, panel on the right) was transplanted with 11,598 IEQ/kg and 0.1 x 10⁹ DBMC/kg from a mismatched donor into the liver on POD 0, followed by IV DBMC (0.33 x 10⁹ cells/kg) on POD 5 and 11. The animal from group 2 (35-493, panel on the left) was transplanted with 10,978 IEQ/kg and 1.4 x 10⁶ MSC/kg from a mismatched donor into the liver on POD 0, followed by IV donor MSC (5.8 x 10⁶ cells/kg) together with CD11b depleted donor bone marrow cells (DBMC, 0.30 x 10⁹ cells/kg) on POD 5 and 11. Fasting c-peptide levels for recipients of allogeneic islets in the liver at 3 days, 2 weeks and 1 month posttransplant. Empty bars represent recipients of islet + MSC codelivery in the liver and delayed IV donor bone marrow (DBMC)+ MSC infusion ( n=8; group 2, table 2). Black bars
Figure 5: Exogenous insulin requirements (EIR) and fasting blood glucose (FBG) (upper panel) and fasting c-peptide (lower panel) for animal 26-20. This animal received induction therapy with thymoglobulin and fludarabine in the week prior to transplant, and IM rapamycin was initiated on POD 14 to achieve and maintain trough levels of 15-20 ng/ml. In addition, treatment with anti-CD154 (20 mg/kg) started on POD -1, with 5 doses in the first month posttransplant and monthly thereafter until POD 168. On POD 0, 3,928 IEQ/kg and 1.6 x 10^6 MSC/kg from a mismatched donor were transplanted into the liver. IV donor MSC (5.3 x 10^6 cells/kg), together with CD11b depleted donor bone marrow cells (0.20 x 10^9 cells/kg) were given on POD 5 and 11. Additional donor MSC (full arrow, 2 x 10^6 cells/kg) were given on POD 64 and 68; and 3rd party MSC from a haploidentical 3rd party (dashed arrow, 2 x 10^6 cells/kg) were given on POD 71, 77, 86, 91, 155 and 160.

Figure 6: A. Exogenous insulin requirements (EIR) and fasting blood glucose (FBG) (upper panel) and fasting c-peptide (lower panel) for animal 105-131. This animal received induction therapy with thymoglobulin and fludarabine in the week prior to transplant, and IM rapamycin was initiated on POD 14 to achieve and maintain trough levels of 15-20 ng/ml. In addition, treatment with anti-CD154 (20 mg/kg) started on POD -1, with 5 doses in the first month posttransplant and monthly thereafter. On POD 0, 3,000 IEQ/kg and 1.0 x 10^6 MSC/kg from a haploidentical donor were transplanted into the liver. IV donor MSC (3.4 x 10^6 cells/kg), together with CD11b depleted donor bone marrow cells (0.17 x 10^9 cells/kg) were given on POD 5 and 11. Additional donor MSC (full arrow, 2 x 10^6 cells/kg) were given on POD 105, 110, 196 and 207. B. Exogenous insulin requirements (EIR, line), % CD4/25 bright FoxP3 T cells (filled circles), and %CD3/8 positive T cells (empty circles) for animal 105-131. Arrows indicate donor MSC (2 x 10^6 cells/kg) given on POD 105, 110, 196 and 207. C. Frequency of FoxP3 positive T cells in peripheral blood of animal 105-131 gated for CD3^+CD4^+CD25^bright^ lymphocytes. D. Histopathology of liver sections after necropsy. Left panel shows immunohistochemistry staining of an islet with hematoxilin-eosin and positive signal for insulin (brown) (200X). Right panel shows a representative confocal microscopy analysis with immunofluorescence for insulin (red), glucagon (blue) and blood vessels (green) (400X).
Figure 1

A

% Positive

CD105 CD29 CD73 Class I CD90 CD44 CD166 CD56 CD45 CD14 Class II CD11c CD34 CD31

B

Count

CD105

Class I

CD29

CD73

CD45

CD31

C

Adipogenic Control

Osteogenic Control

D

Average CPM

no MSC (n=5) Recipient MSC (n=2) Allogeneic MSC (n=13)
Figure 2

MSC and islet engraftment in nonhuman primates

HGF

GALECTIN-1

IL-6

VEGF

TGF-β

IL-10

Log \{Target Gene/18S\} × 10000

Log \{(IL10/18S) × 10^(-8)}

Passage

PBL     P0     P1     P2     P3      P4     P5

124-1280  85-740  96Y  105-98  205Y  105-74  86Z  13-578
Figure 3

P0

P2

P6

P11
Figure 4

A

Group 1 (n=3)

Day
-6 -4 -3 -2 -1 0 3 5 10 11 14 18 28 49 monthly

Thy + Flu
Rapa
IP islets + DBMC
IV DBMC

Group 2 (n=8)

Day
-6 -4 -3 -2 -1 0 3 5 10 11 14 18 28 49 monthly

Thy + Flu
* 5C8
* Daily PTH
* Rapa
* Delayed Rapa
IP islets + MSC
IV DBMC + MSC

B

Islets/MSC

<table>
<thead>
<tr>
<th>EIR (IU/kg/day)</th>
<th>Fasting Blood Glucose (mg/dl)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
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<td>2</td>
<td>200</td>
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<td>3</td>
<td>300</td>
</tr>
<tr>
<td>4</td>
<td>400</td>
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</table>

C

<table>
<thead>
<tr>
<th>C-peptide (ng/ml)</th>
<th>MSC IP</th>
<th>DBMC IP</th>
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<tbody>
<tr>
<td>3 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 month</td>
<td></td>
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</tr>
</tbody>
</table>

* indicates significant difference.
Figure 5

- **EIR (IU/kg/day)**
- **FBG (mg/dl)**
- **C-peptide (ng/ml)**

POD:
- 0
- 50
- 100
- 150
- 200

EIR (IU/kg/day):
- 0
- 1
- 2
- 3
- 4

FBG (mg/dl):
- 0
- 100
- 200
- 300
- 400

C-peptide (ng/ml):
- 0
- 1
- 2
- 3
- 4
- 5
- 6
Figure 6

A

B

C

D